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Master Thesis

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Abstract

MLinvitroTox employs hazard-driven prioritization to rapidly rank compounds based on their predicted toxicities in specific endpoints, thus circumventing the resource-intensive process of chemical compound identification within complex environmental samples..

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Chapter 1

Introduction

1.1 The Challenge of Environmental Pollution

Over the past few decades, the upsurge in environmental pollution by chemical compounds has been driven by industrial processes, agricultural methods, consumerism and various other contributing factors. Although these chemicals are integral for many products and have the potential to improve the comfort of modern society, they can also pose risks and adversely affect both human health and the environment, either acutely or chronically. Toxic substances threaten wildlife but also make air, soil, drinking water and food supply less safe.

Nations worldwide maintain comprehensive chemical regulations¹, however, it is anticipated that global chemicals production will double by 2030 [1]. Moreover, the widespread utilization of chemicals, including their inclusion in consumer goods, is expected to expand further. Even though there are over 275 million known chemical compounds registered by the *Chemical Abstracts Service* [2], merely a tiny fraction of them undergo close monitoring via target analytical approaches and even less is known about their toxicity profiles and negative health effects on organisms. Refer to Table 1.1 for an overview of omnipresent water pollutants.

In light of the rapidly evolving chemical landscape, there is an increasing demand for future-proof, robust measurement and modeling methods. These methods are essential for evaluating the toxicity and exposure of chemicals, facilitating informed risk-based decision-making even when data on hazards and exposures are limited. It is worth noting that the need for adaptable approaches in chemical safety and sustainability efforts must also prioritize cost-efficiency and gain widespread acceptance among regulatory bodies, industry stakeholders, and the general public.

For instance, the EU has introduced the 8th Environment Action Programme, as out-

¹For instance, REACH, short for Registration, Evaluation, Authorisation, and Restriction of Chemicals, is an EU regulation aimed at improving chemical safety and allocating risk management responsibilities to companies operating in various sectors.

1.2. The Imperative for Prioritization and Toxicity Assessment

lined in its European Green Deal ([citeregreendeal](#)), to provide direction for European environmental policy until the year 2030. This program reinforces the EU's ambitious goal of sustainable living within planetary limits, with a forward-looking vision that extends to 2050. Central to this vision is a zero-pollution commitment, encompassing air, water, and soil quality, all while prioritizing the well-being of EU citizens. In 2021, the European Commission introduced a sustainability-focused chemicals strategy [4], which aligns with the EU's zero-pollution ambition. This strategy not only enables the evaluation of the safety and sustainability of both existing and future chemical compounds but also aims to reduce concerning substances, such as *per- and polyfluoroalkyl substances (PFAS)*, through substitution or phasing out wherever feasible. In parallel, the U.S. Environmental Protection Agency (EPA) shares a similar scientific consensus and is at the forefront of assessing the potential impacts of chemicals on human health and the environment. Leveraging advanced toxicological and exposure methods, EPA actively promotes risk reduction efforts through its own Chemical Safety for Sustainability National Research Program. This program builds upon the achievements of research initiatives like *ToxCast*², *Tox21*³, and the Endocrine Disruptor Screening Program in the 21st Century (EDSP21), demonstrating a commitment to advancing chemical safety on a global scale.

1.2 The Imperative for Prioritization and Toxicity Assessment

Modern analytical techniques, including *high resolution mass spectrometry (HRMS/MS)*, are gaining significance across various domains such as metabolomics, drug discovery, environmental science and toxicology [5].

In environmental monitoring, the application of nontarget HRMS/MS has notably improved the capacity to detect possibly thousands of contaminants in a single sample. The instrument generates complex spectra that provide information about the masses and fragmentation patterns of compounds present within the sample as illustrated in Figure 1.1. Often, only a minority of these molecules can be definitively identified, while the majority remains unidentified, resulting in their classification into two categories:

- **Identified compounds** are substances for which their chemical structure and properties have been determined and confirmed using additional analytical techniques. These compounds are precisely characterized and can be linked to existing databases or reference spectra, enabling the retrieval of information about their toxicity and other relevant characteristics.
- **Unidentified compounds**, on the other hand, are substances that are detected but lack definitive characterization in terms of their chemical identity, structure, or properties, including its toxicity. Unidentified compounds are observed as peaks or features in these spectra, but their specific chemical attributes remain

²<https://www.epa.gov/comptox/toxcast>

³<https://tox21.gov/>

1.2. The Imperative for Prioritization and Toxicity Assessment

unknown. Further examination of these compounds is necessary, but it entails a substantial investment of time and resources, emphasizing the importance of prioritization.

When it comes to prioritizing unidentified compounds for further comprehensive testing, the standard approach has been to rely on signal intensity from fragmentation data. However, this approach tends to fall short in delivering an accurate assessment of environmental exposures because the signal intensity may not relate proportionally to the compound's concentration in the sample. Furthermore this approach overlooks the toxicological factors essential for prioritizing compounds with concerns related to environmental hazards. As a result, substances with the potential for severe ecological consequences, such as endocrine-disrupting compounds, often go undetected because of their low abundance, even though they exhibit high levels of toxicity. Hence, a pressing need exists for alternative approaches to prioritize unidentified nontarget HRMS/MS signals based on their hazard potential. By incorporating relevant toxicity factors into the equation:

$$\text{Risk} = \text{Hazard} \times \text{Exposure} \quad (1.1)$$

we augment the capacity to make well-informed decisions when evaluating the environmental risk associated with chemicals.

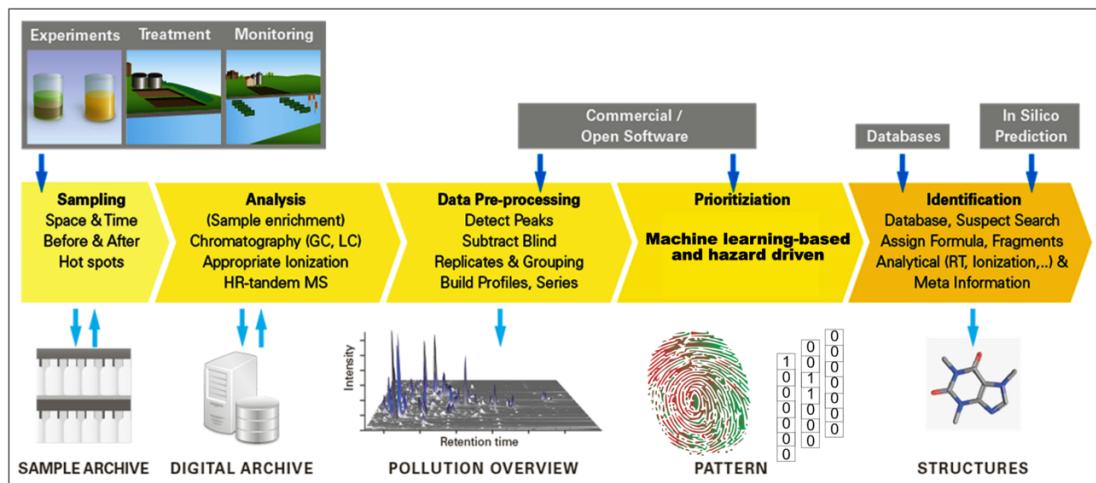


Figure 1.1: Schematic of the workflow used for nontarget HRMS/MS screening of environmental samples, featuring a customized prioritization step. Adapted from Figure 1 in the original source [6].

1.3. Unlocking the Potential of High-Throughput Screening and Machine Learning in Toxicity Prediction

1.3 Unlocking the Potential of High-Throughput Screening and Machine Learning in Toxicity Prediction

In the past few years, the use of machine learning methods has emerged as a transformative force in the field of *in vitro* toxicology, particularly in the realm of high-throughput toxicity prediction. *High-throughput screening (HTS)* has revolutionized the way toxicity is assessed by allowing thousands of *in vitro* bioassays to be conducted efficiently. This high-throughput approach, coupled with advancements in robotics and automated analysis, has generated large volumes of toxicity data, paving the way for more comprehensive assessments of chemical compounds. Alongside the rise of machine learning, this advancement has facilitated the creation of predictive models, known as *Quantitative structure-activity relationship (QSAR)* models. These models are capable of forecasting bioactivity or compound toxicity based on their physico-chemical properties or molecular descriptors [7]. As they are trained on extensive datasets containing comprehensive toxicity information, these models can learn the underlying patterns and relationships between chemical structures and target toxicity. With this capability, they can predict the toxicity of new compounds, even when these substances themselves have not undergone laboratory testing. This approach holds the potential to substantially decrease the time and expenses linked to initial toxicity pre-assessment, and it plays a pivotal role in determining which compounds should undergo more in-depth testing.



(a) A robot arm retrieves assay plates from incubators and places them at compound transfer stations or hands them off to another arm that services liquid dispensers or plate readers. Efforts in the automation, miniaturization and the readout technologies have enabled the growth of HTS. Image obtained from [8].

(b) Modern microtitre assay plates consist of multiples of 96 wells, which are either prepared in the lab or acquired commercially from stock plates. These wells are filled with a dilution solvent, such as *Dimethylsulfoxide (DMSO)*, along with the chemical compounds intended for analysis. Image obtained from [9].

Figure 1.2: High-Throughput Screening (HTS)

1.4 MLinvitroTox: A Novel Approach

In response to the pressing need for a more hazard-driven and comprehensive assessment of environmental contaminants, Arturi *et al.* introduced *MLinvitroTox* [10], an innovative machine learning framework. This framework is part of a broader pipeline named *EXPECTmine*, which incorporates the complementary exposure aspect within the risk assessment process. The primary objective of this thesis is to collaborate with the authors to further enhance and advance this framework. *MLinvitroTox* leverages molecular fingerprints extracted from fragmentation spectra, marking a significant change in how the toxicity of the myriad unidentified HRMS/MS features is forecasted. *MLinvitroTox* follows a similar training approach as traditional QSAR models, using supervised classification models trained with molecular fingerprints derived from chemical structures. However, during the application phase, the input to the machine learning model consists of molecular fingerprints generated from experimentally measured MS2 spectra using *SIRIUS* and *CSI:FingerID* [11]. *SIRIUS* is a software package for annotating small molecules from nontarget HRMS/MS data, while *CSI:FingerID* is a machine-learning tool employed by *SIRIUS* to predict molecular fingerprints from fragmentation spectra. Utilizing streamlined machine learning methodologies, *MLinvitroTox* forecasts chemical toxicity for a wide range of compounds. This comprehensive analysis covers more than 400 target-specific and 70 cytotoxic endpoints, drawing data from ToxCast/Tox21 datasets. Subsequently, the toxicity predictions generated by the framework are employed to prioritize compounds, with the flexibility to emphasize specific aspects of toxicity profiles tailored to individual preferences.

1.5 Objectives and Significance

The main objective of this thesis is to contribute to the development of an efficient *MLinvitroTox* framework for predicting compound toxicity across multiple endpoints. The goal is to enhance the integration of *MLinvitroTox* by creating an automated pipeline in the Python programming language. This pipeline is designed to efficiently address the inherent complexities associated with modeling and processing heterogeneous datasets. Here, the emphasis lies in enhancing the curation and filtering of toxicological data and streamlining the process, which begins with raw concentration-response series data and culminates in the generation of the final toxicity predictions. The ultimate output is expected to comprise toxicity fingerprints that encapsulate the predicted toxicity from HRMS/MS environmental samples for the relevant endpoints of interest. These generated toxicity fingerprints will offer crucial insights for the prioritization process, aiding in the identification of the most hazardous compounds present in environmental samples.

One notable constraint of the existing framework lies in its binary *hitcall* when predicting the toxicity of specific endpoints. It categorizes compounds as either toxic or non-toxic without accounting for variations in toxicity severity. In the long term, it is crucial to adopt a more refined approach that can capture the nuanced continuum of toxicity.

This thesis endeavors to overcome this limitation by developing a pipeline capable of forecasting toxicity across numerous endpoints, employing continuous hitcalls.

1.6 Thesis Structure

In the course of progressing through the subsequent chapters, insights will be provided into the materials and methods employed, focusing on the technical intricacies involved in the preparation of ToxCast/Tox21 toxicity data and their transformation into suitable inputs for the machine learning pipeline. This foundational work will establish the basis for the upcoming chapters, which will showcase the potential of MLinvitroTox. Furthermore, the framework's effectiveness is demonstrated through the validation of real-world mass spectral data from *MassBank* [12], and the examination of the implications of this research is carried out.

1.6. Thesis Structure

Origin/Usage	Class	Examples	Related Issues
Industrial Chemicals	Solvents	Tetrachloro-methane	Drinking-water-quality
	Intermediates	Methyl-t-butylether	Drinking-water-quality
	Petrochemicals	BTEX (benzene, toluene, xylene)	Cancer
Industrial Products	Additives	Phthalates	Endocrine disruptors
	Lubricants Flame Retardants	PCBs PBDEs	Biomagnification
Consumer Products	Detergents	Nonylphenol ethoxylates	Endocrine effects
	Pharmaceuticals	Antibiotics	Bacterial resistance
	Hormones	Ethinyl estradiol	Feminization of fish
Biocides	Pesticides	DDT	Toxic effects and persistent metabolites
	Nonagricultural biocides	Tributyltin	Endocrine effects
Geogenic & Natural Chemicals	Heavy Metals	Lead, cadmium, mercury	Organ damage
	Inorganics	Arsenic, selenium, fluoride	Drinking-water-quality
	Taste and Odor	Geosmin	
	Human Hormones	Estradiol	Feminization of fish
Disinfection & Oxidation	Disinfection by-products	Haloacetic acids, Bromate	Drinking-water-quality
Transformation Products	Metabolites from all above	Metabolites of perfluorinated compounds Chloroacetanilide herbicide metabolites	Bioaccumulation Drinking-water-quality

Table 1.1: Examples of ubiquitous water pollutants. Table 2 adapted from [3].

Chapter 2

Background

This chapter is vital for understanding the following sections of this thesis as it provides some foundational background information in toxicity testing.

2.1 Toxicity Testing: From In Vitro Assays and Molecular Fingerprints to Predictive Models and Beyond

With the ever-growing amount of chemical compounds entering the environment, traditional experimentation methods face limitations concerning cost and time constraints. Additionally, ethical concerns arise regarding the use of animal trials in *in vivo* experiments.

In 2007, the *U.S. National Academy of Sciences* introduced a visionary perspective and published a landmark report, titled as *Toxicity Testing in the 21st Century: Vision and Strategy*. This report promoted a transition from conventional, resource-consuming animal-based *in vivo* tests to efficient high-throughput *in vitro* pathway assays on cells. This transition paved the way for the realm of HTS, where a multitude of *in vitro* bioassays can be executed, complementing and improving chemical screening. This transformation is made possible by advancements in robotics, data processing, and automated analysis. As a result, this synergy has led to the generation of extensive toxicity datasets like ToxCast and Tox21.

HTS datasets, including ToxCast and other sources, have opened the door to promising applications of machine learning in predictive computational toxicology. These predictive models can be developed to screen environmental samples with limited availability of toxicity data, allowing for the prioritization of further testing efforts. Such models often forecast toxicity using QSARs, which are based on descriptors encoding chemical structures like molecular fingerprints. 1D-Molecular fingerprints encode compound molecules as fixed-length binary vectors, denoting the presence (1) or absence (0) of specific substructures or functional groups.

2.1. Toxicity Testing: From In Vitro Assays and Molecular Fingerprints to Predictive Models and Beyond

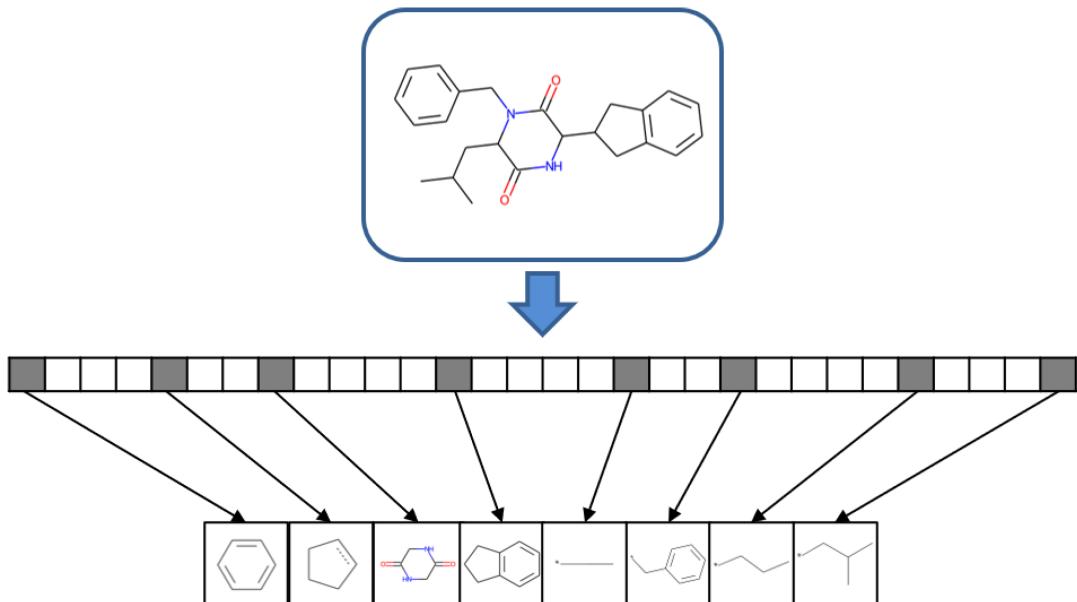


Figure 2.1: Schematic of a molecular fingerprint for a fictional chemical. Each bit position accounts for the presence or absence of a specific structural fragment. Bit positions are set on (set to 1, gray) if the substructure is present in a molecule, or set off (set to 0, white) if it is absent. Figure 1 adapted from [13].

The utilization of molecular fingerprints for *in vitro* toxicity prediction is based on the assumption that molecular toxic effects result from interactions between distinct chemical components and receptors during a *molecular initiating event (MIE)*. On a larger biological scale, the MIE can set a sequential chain of causally linked *key events (KE)* in motion. This occurs at different levels of biological organisation from within cells to potentially culminating in an *adverse outcome pathway (AOP)* at the organ or organism level, as depicted in Figure 2.2. The mechanistic information captured in AOPs reveal how chemicals or other stressors cause harm, offering insights into disrupted biological processes, potential intervention points but also guide regulatory decisions on next generation risk assessment and toxicity testing. The AOP framework is an analytical construct that allows an activity mapping from the presence or absence of certain molecular substructures encoded in chemical descriptors to the target mechanistic toxicity. Finally, when monitoring disruptions in toxicity pathways, physiologically based pharmacokinetic (PBPK) models can be leveraged to extrapolate *in vitro* findings to human blood and tissue concentrations [14].

It is crucial to emphasize that the predictions from HTS bioassays portray molecular toxicity events only at a cellular level, and their translation to adverse outcomes at higher organism levels is not necessarily guaranteed. As the scale shifts from the cellular to the organism level, the confidence in these relationships may decrease.

2.2. Chemical Target Toxicity vs. Cytotoxicity

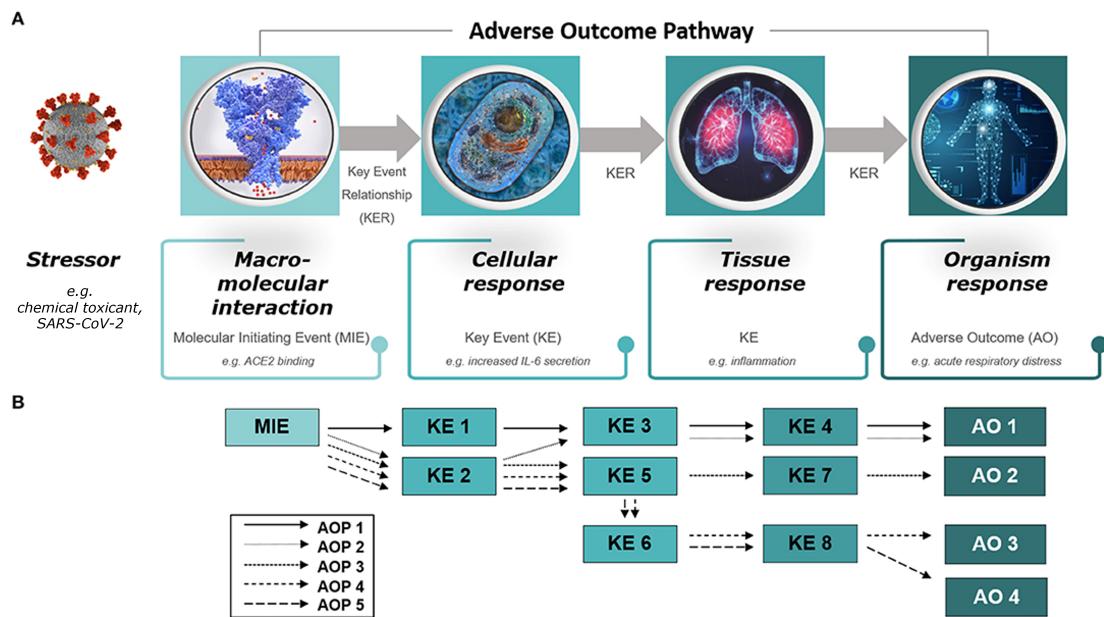


Figure 2.2: Diagram of (A) an adverse outcome pathway (AOP) and (B) an AOP network. (A) An AOP starts with a molecular initiating event (MIE), followed by a series of key events (KEs) on different levels of biological organization (cellular, tissue, organ) and ends with an adverse outcome (AO) in an organism. The stressor is not part of the AOP itself. Figure 1 adapted from [15]

2.2 Chemical Target Toxicity vs. Cytotoxicity

Consider a hypothetical scenario in which a chemical undergoes testing in a bioassay that assesses toxicity by measuring the activation of a *reporter gene* within a cell. The reporter gene encodes a detectable protein, and its activation is triggered by the chemical binding to a specific receptor, the key focus of the assay endpoint. The resulting signal is proportional to the chemical's concentration. While it might seem logical that an increase in chemical concentration would result in higher chemical toxicity, this assumption does not always hold true. At elevated concentrations, the chemical can become *cytotoxic*, causing harm to the cells and ultimately leading to cell death. Consequently, this can lead to a decrease in the activation of the reporter gene and a subsequent reduction in the signal, indicating a decrease in bioactivity. For a visual representation, please refer to Figure 2.3. Considering this situation, chemical toxicity can manifest in various forms, categorizing into two primary groups [16]:

1. **Specific toxicity** is the result of a chemical's interaction and disruption of a specific biomolecular target or pathway, such as a receptor agonist/antagonist effect or enzyme activation/inhibition. This work is primarily concerned with specific toxicity. However, it is essential to recognize that data processing must also take into account the following:
2. **Non-specific toxicity (Cytotoxicity and cell stress)** involve broad disruptions of

2.2. Chemical Target Toxicity vs. Cytotoxicity

the cellular machinery, including reactions with DNA as well as processes like apoptosis, oxidative stress and mitochondrial disturbance. Cell viability can be evaluated either individually or concurrent with the target bioassay endpoint. For instance, one approach involves evaluating the cell viability by determining the proportion of live cells within a population. This is achieved using a fluorescent dye that selectively enters living cells, as it cannot permeate the membranes of deceased cells, resulting in fluorescence intensity directly reflecting cell viability.

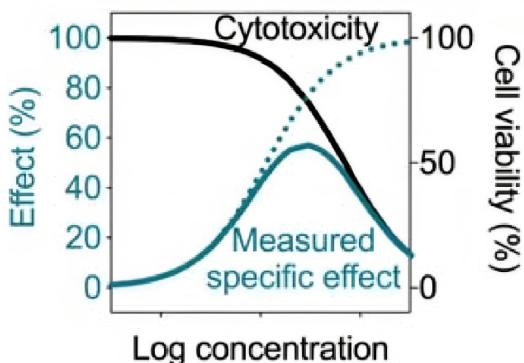


Figure 2.3: Example of a bioassay response with cytotoxicity interference. The dotted line shows the theoretical effect but due to cytotoxicity (black line is cell viability), the measured effect has an inverted U-shape. The measured effect can additionally be confounded by the cytotoxicity burst, where even an exponential curve shape is likely for the gaining part. Figure 7.8 from [17].

An associated phenomenon is referred to as the *cytotoxicity burst* [16], in which the expected specific toxicity interferes with non-specific cellular stress responses that may become overly activated within a critical range of toxicant concentration. As the concentration of the toxicant approaches levels that cause cell death, the signal measuring the supposed specific toxicity of a target assay endpoint becomes mixed with signals from non-specific responses [17]. Compounds that attain an efficacy response exceeding the toxicity threshold within the tested concentration range solely due to these non-specific responses are termed *false positive* hitcalls. This introduces uncertainty about the reliability of reported activity hitcalls, and false positive hitcalls can arise without a comprehensive evaluation of cytotoxicity interference.

The ToxCast pipeline is intentionally designed to minimize *false negative* hitcalls by adopting an inclusive risk assessment approach, ensuring that potentially toxic compounds are not overlooked. Nevertheless, the occurrence of false positive hitcalls can be mitigated through a comparison of potency concentrations between the target assay endpoints and the respective viability or burst assay endpoints that quantify cytotoxic cell loss or cell stress. If the probabilities suggest that the potency concentration of the cytotoxicity assay endpoint is lower than that of the target assay endpoint, previously identified false positive hitcalls can be reevaluated as potential instances of cytotoxicity interference.

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